Disruption of Abscisic Acid Signaling Constitutively Activates Arabidopsis Resistance to the Necrotrophic Fungus *Plectosphaerella cucumerina*

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Plant resistance to necrotrophic fungi is regulated by a complex set of signaling pathways that includes those mediated by the hormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA), and abscisic acid (ABA). The role of ABA in plant resistance remains controversial, as positive and negative regulatory functions have been described depending on the plant-pathogen interaction analyzed. Here, we show that ABA signaling negatively regulates Arabidopsis (*Arabidopsis thaliana*) resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Arabidopsis plants impaired in ABA biosynthesis, such as the *aba1-6* mutant, or in ABA signaling, like the *quadruple pyr/pyl* mutant (*pyr1pyl1pyl2pyl4*), were more resistant to *P. cucumerina* than wild-type plants. In contrast, the *hab1-1abi1-2abi2-2* mutant impaired in three phosphatases that negatively regulate ABA signaling displayed an enhanced susceptibility phenotype to this fungus. Comparative transcriptomic analyses of *aba1-6* and wild-type plants revealed that the ABA pathway negatively regulates defense genes, many of which are controlled by the SA, JA, or ET pathway. In line with these data, we found that *aba1-6* resistance to *P. cucumerina* was partially compromised when the SA, JA, or ET pathway was disrupted in this mutant. Additionally, in the *aba1-6* plants, some genes encoding cell wall-related proteins were misregulated. Fourier transform infrared spectroscopy and biochemical analyses of cell walls from *aba1-6* and wild-type plants revealed significant differences in their Fourier transform infrared spectratypes and uronic acid and cellulose contents. All these data suggest that ABA signaling has a complex function in Arabidopsis basal resistance, negatively regulating SA/JA/ET-mediated resistance to necrotrophic fungi.

Plants are exposed in their natural environments to biotic and abiotic stresses. Under these conditions, plant survival depends on their ability to detect stress-associated signals and to respond to these stimuli quickly and efficiently (Bari and Jones, 2009). The mechanisms involved in regulating the activation of defensive responses upon biotic and abiotic stresses are not fully understood. Intricate networks involving different signaling pathways are required for the activation of specific plant responses to a particular stress, but how these pathways interact to balance the final output response is largely unknown (Fujita et al., 2006; Robert-Seilaniantz et al., 2007; Spoel and Dong, 2008; Bari and Jones, 2009). Plant resistance to pathogens depends on the interplay of different signaling mechanisms, such as those mediated by the hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Thomma et al., 1998; Glazebrook, 2005). In addition to these well-characterized pathways, other plant hormones, such as abscisic acid (ABA), brassinosteroids, gibberellins, and auxins, are emerging as important coregulators of plant resistance to pathogens, including necrotrophic fungi (Bari and Jones, 2009).

ABA regulates many aspects of plant development, such as seed dormancy and germination, and also controls plant responses to abiotic and biotic stress (Fujita et al., 2006; Wasilewska et al., 2008). The mechanism of action of at least one arm of the ABA perception/signaling pathway has been recently characterized at the molecular level (Raghavendra et al., 2010). The activated ABA receptor was demonstrated to be a heteromeric

complex formed by an ABA-binding RCAR/PYR1/PYL family member (for Regulatory Component of ABA Receptor/Pyrabactin Resistance1/PYR1-Like) and a "clade A" type 2C protein phosphatase (PP2C; Schweighofer et al., 2004) such as Abscisic Acid Insensitive1 (ABI1), ABI2, or Hypersensitive to ABA1; Ma et al., 2009; Park et al., 2009). In the presence of ABA, the receptor blocks the phosphatase activity of PP2Cs; consequently, protein kinases, such as OST1 and SnRKs, are no longer inhibited, and they phosphorylate key targets of the ABA signaling pathway (Umezawa et al., 2009; Vlad et al., 2009).

The role of ABA signaling in the regulation of plant basal resistance to pathogens is complex and not completely understood. For example, in plant resistance to necrotrophic pathogens, positive and negative actions have been described for ABA depending on the pathosystem studied, the plant developmental stage tested, or the environmental conditions used for plant growth (Mauch-Mani and Mauch, 2005; Ton et al., 2009; García-Andrade et al., 2011; Robert-Seilaniantz et al., 2011). In tomato (*Solanum lycopersicum*), a negative regulatory role of ABA in resistance to necrotrophic pathogens, such as the fungus *Botrytis cinerea* and the bacterium *Dickeia dadantii*, has been demonstrated. These pathogens are less virulent on the tomato *sitiens* mutant, in which ABA biosynthesis is disrupted, than in wild-type plants (Audenaert et al., 2002; Asselbergh et al., 2007, 2008a, 2008b). Similarly, Arabidopsis (*Arabidopsis thaliana*) ABA-deficient mutants (e.g. *aba2* and *aao3*) are more resistant than wild-type plants to the necrotroph *B. cinerea* and to the vascular fungal pathogen *Fusarium oxysporum* (Audenaert et al., 2002; L'Haridon et al., 2011). Several molecular mechanisms were proposed to explain the enhanced resistance phenotype of Arabidopsis and tomato ABA-deficient mutants. For example, in tomato *sitiens* plants, enhanced up-regulation of defense-related transcripts (e.g. *PR-1*), accumulation of reactive oxygen species (ROS), and increase in cuticle permeability were suggested to enhance resistance to *B. cinerea* and *D. dadantii* (Audenaert et al., 2002; Asselbergh et al., 2007; Curvers et al., 2010). Similarly, enhanced resistance of Arabidopsis ABA-deficient mutants (*aba2*) to *B. cinerea* was linked to increased cuticle permeability, which was also predicted to account for the stronger and faster accumulation of ROS seen following fungal infection (L'Haridon et al., 2011).

Exogenous application of ABA to Arabidopsis wild-type plants resulted in an enhanced susceptibility to *Pseudomonas syringae* pv *tomato* DC3000 that was correlated with inhibition of lignin biosynthesis, reduced SA accumulation, and suppression of transcripts of several defense-related genes, including some regulated by SA and JA/ET signaling pathways (Anderson et al., 2004; Mohr and Cahill, 2007). Treatment of Arabidopsis with ABA also blocked the activation of systemic acquired resistance (SAR) both upstream and downstream of SA biosynthesis (Yasuda et al., 2008). Congruently, SAR activation disrupted the up-regulation of ABA biosynthesis and signaling in plants exposed to salt stress, further indicating a negative cross talk between SAR and ABA signaling pathways (Yasuda et al., 2008). Plant pathogens also utilize ABA-mediated suppression of plant defensive responses to colonize plant tissues. The AvrPtoB effector of *P. syringae* pv *tomato* DC3000 induces the accumulation of ABA in Arabidopsis to suppress the induction of basal defense by down-regulating SA biosynthesis and signaling pathways (de Torres-Zabala et al., 2007, 2009). Further support for an important role for ABA-SA cross talk in plant defense comes from a recent study demonstrating that activation of *EDS1/PAD4*-dependent immune responses rapidly disrupts ABA signaling transduction (Kim et al., 2011).

ABA also has a positive role in the regulation of Arabidopsis basal resistance to certain pathogens. Increased susceptibility to the necrotrophic fungus *Alternaria brassicicola*, the vascular oomycete *Pythium irregulare*, and the necrotrophic fungus *Plectosphaerella cucumerina* was observed in ABA-deficient mutants (Adie et al., 2007; Flors et al., 2008; García-Andrade et al., 2011). ABA is required for JA biosynthesis and induction of the expression of JA-regulated defense genes, two essential processes for full Arabidopsis resistance to *P. irregulare* (Adie et al., 2007). ABA triggers callose deposition upon *A. brassicicola* infection, a defense mechanism associated with restriction of colonization by the fungus. A reduction of ABA levels in plants infected with *A. brassicicola* may represent a virulence defense mechanism evolved to favor colonization (Flors et al., 2008). A role for ABA in mimicking β-aminobutyric acid-primed callose deposition and some defense responses against the necrotrophic pathogens *A. brassicicola* and *P. cucumerina* has also been described (Ton and Mauch-Mani, 2004; Flors et al., 2005; García-Andrade et al., 2011). Moreover, the enhanced and constitutive accumulation of ABA of the Arabidopsis *irx1-6* mutant, impaired in a secondary cell wall cellulose synthase (AtCESA8), and of the *ocp3* mutant, defective in a transcriptional factor of the homeodomain family, has been suggested to contribute to increased resistance to several pathogens (Hernández-Blanco et al., 2007; García-Andrade et al., 2011).

In this work, we show that disruption of ABA biosynthesis or signaling leads to a constitutive activation of Arabidopsis defensive response. We provide genetic evidence that supports a negative function of ABA in the regulation of SA/ET/JA-dependent immune responses, which are essential for Arabidopsis resistance to the virulent, necrotrophic fungal strain *P. cucumerina Brigitte Mauch-Mani* (*PcBMM*). Moreover, we show that the *aba1-6* mutant displays alterations in its cell wall structure and composition.

RESULTS

ABA-Deficient and Signaling Mutants Are More Resistant Than Wild-Type Plants to the Necrotrophic Fungus *P. cucumerina BMM*

To investigate the role of ABA in the regulation of Arabidopsis resistance to necrotrophic fungal pathogens,

we tested the susceptibility to *PcBMM* of plant mutants impaired in ABA biosynthesis, like *aba1-6* (Niyogi et al., 1998), in ABA signaling, such as in *quadruple pyr/pyl* (*pyr1pyl1pyl2pyl4*; Park et al., 2009), or displaying a partial constitutive activation of the ABA pathway, as occurs in the *triple abi2-2* mutant *hab1-1abi1-2abi2-2* (Rubio et al., 2009). We inoculated 3-week-old ecotype Columbia (Col-0) wild-type plants and the ABA mutants with a spore suspension (4×10^6 spores mL^{-1}) of the *PcBMM* isolate that causes disease in a broad range of Arabidopsis accessions (Llorente et al., 2005; Sánchez-Vallet et al., 2010). Progression of the infection was examined at different hours/days post inoculation (hpi/dpi) by determining fungal growth and plant cell death, using trypan blue (TB) staining of inoculated leaves, the fungal biomass using quantitative PCR (qPCR) of the *PcBMM β-Tubulin* gene, and by macroscopic evaluation of disease rating (DR; Sánchez-Vallet et al., 2010). As shown in Figure 1, *aba1-6* and *quadruple pyr/pyl* mutant supported lower fungal growth than wild-type plants, as revealed by TB staining of inoculated leaves 1 dpi and by fungal biomass quantification 3 dpi (Fig. 1, A and C). In contrast, fungal growth in the *triple abi2-2* mutant was higher than that determined in Col-0 plants (Fig. 1, A and C). These results were corroborated by confocal microscopy analyses of Col-0 and *aba1-6* plants inoculated with *PcBMM-GFP*, a fungal transformant constitutively expressing GFP (Supplemental Fig. S1; Ramos et al., 2012). A positive correlation was found between in planta fungal biomass, plant cell death, and macroscopic disease symptoms (Fig. 1, A, B, and D; data not shown). Thus, the level of plant cell death, as determined by TB staining, was lower in *aba1-6* and *quadruple pyr/pyl* mutants than in wild-type plants, whereas it was enhanced in the *triple abi2-2* mutant (Fig. 1D). Accordingly, the *aba1-6* mutant, which has a dwarf phenotype (Barrero et al., 2005), and the *quadruple pyr/pyl* mutant displayed similar, less severe macroscopic symptoms than those observed in inoculated wild-type plants, whereas the opposite was true for the *triple abi2-2* mutant (Fig. 1B; data not shown). These data strongly supported a negative function of the cytosolic ABA perception and signaling pathways in the regulation of Arabidopsis resistance to *PcBMM* and further corroborated the relevant function of the ABA pathway in the control of Arabidopsis resistance to necrotrophic fungal pathogens.

ROS regulates Arabidopsis responses to biotic and abiotic stresses (Torres et al., 2005). ABA has been suggested to negatively regulate ROS accumulation in Arabidopsis and tomato in resistance responses to the necrotrophic fungus *B. cinerea* (Asselbergh et al., 2007; Curvers et al., 2010; L'Haridon et al., 2011). Therefore, we used 3,3′-diaminobenzidine (DAB) staining to determine ROS production in *PcBMM*-inoculated wild-type plants and in the three ABA mutants, but no significant differences were macroscopically observed in ROS production among the genotypes tested (Fig. 1E), further indicating that ABA-mediated regulation of Arabidopsis resistance to this fungus is independent of ROS production. ABA has also been described to trigger callose deposition upon pathogen infection, and ABA-deficient mutants were shown to be impaired in callose accumulation after inoculation with some fungal pathogens (Ton and Mauch-Mani, 2004; García-Andrade et al., 2011). However, *PcBMM*-infected plants stained with aniline blue displayed lower callose content in the *aba1-6* and *quadruple pyr/pyl* mutants than in wild-type plants (Fig. 1, F and G), indicating that callose deposition is not determining the enhanced resistance phenotype of these mutants. In line with these results, we found that the *pmr4* mutant, impaired in callose deposition (Nishimura et al., 2003), was as susceptible to *PcBMM* as wild-type plants (Supplemental Fig. S2). This *pmr4* phenotype was not due to the previously described enhanced SA content of this mutant (Nishimura et al., 2003), as the susceptibility to *PcBMM* of the *pmr4 sid2-1* double mutant, defective in SA biosynthesis, was similar to that of susceptible *sid2-1* plants (Supplemental Fig. S2). These results further corroborate that, under our inoculation conditions, callose deposition is not an essential defensive barrier against the necrotrophic fungus *PcBMM* and that additional defense mechanisms determine the enhanced resistance phenotype of *aba1-6* and *quadruple pyr/pyl* mutants.

Comparative Transcriptomic Analyses Reveal Constitutive Activation of a Complex Defensive Network in the *aba1-6* Mutant

To further characterize the molecular mechanisms regulating ABA-mediated resistance to necrotrophs, we selected the well-characterized *aba1-6* mutant (Niyogi et al., 1998; Barrero et al., 2005) and performed a comparative transcriptomic analysis of Col-0 and *aba1-6* plants prior to inoculation (time zero) and at 1 d after water treatment (mock) or inoculation with a spore suspension (4×10^6 spores mL^{-1}) of *PcBMM*. Out of the 22,000 probe sets tested in these transcriptomic analyses, 8,687 showed statistically significant differential abundance according to a two-way ANOVA and a Benjamini and Hochberg multiple testing correction ($P \leq 0.01$; GeneSpring 7.2 software). From these probe sets, 213 were differentially regulated in untreated (time zero) *aba1-6* compared with untreated wild-type plants (Supplemental Table S1). In *PcBMM*-inoculated *aba1-6* plants, 497 probe sets were differentially expressed compared with mock-treated plants, whereas 1,474 probe sets were found differentially expressed between *PcBMM*- and mock-inoculated wild-type plants (Supplemental Tables S2 and S3).

The 213 probe sets differentially regulated in the untreated (time zero) *aba1-6* plants (Supplemental Table S1) were analyzed by hierarchical clustering. As shown in Figure 2A, a significant overlap was found between the transcriptional profiles of untreated *aba1-6* and *PcBMM*-inoculated wild-type plants. In agreement, 128 from the 213 probe sets (60%) that were constitutively up- or down-regulated in untreated *aba1-6* plants were found to be also differentially regulated in wild-type plants upon *PcBMM* inoculation

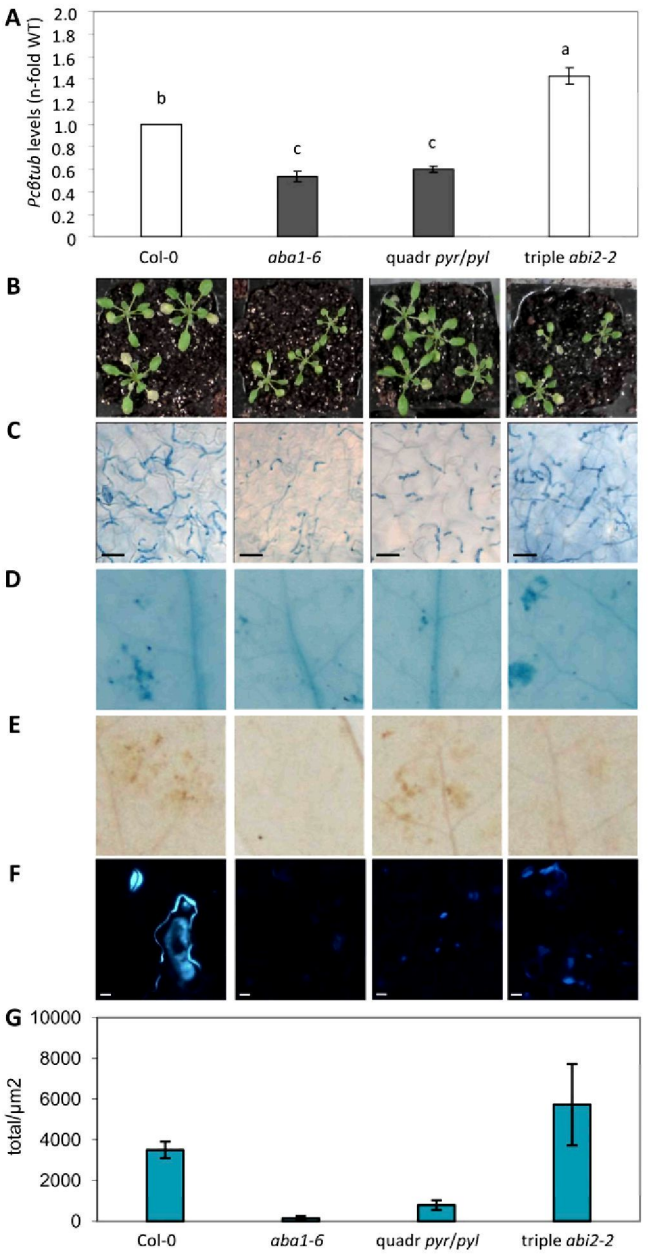

Figure 1. Resistance of ABA-deficient and signaling mutants to the necrotrophic fungus *PcBMM*. Wild-type plants (Col-0; WT) and the *aba1-6*, *quadruple pyr1pyl1pyl2pyl4* (quadr *pyr/pyl*), and *triple abi1-2abi2-2hab1-1* mutants were inoculated with a spore suspension (4×10^6 spores mL^{-1}) of *PcBMM*. A, qPCR quantification of fungal biomass at 3 dpi. Specific primers of *P. cucumerina β-Tubulin* and Arabidopsis *UBIQUITIN21* were used. Values ± SE were normalized to Arabidopsis *UBIQUITIN21* and are represented as averages of the fold increased expression compared with the corresponding wild-type plants. Data represent average values of two replicates from one out of three independent experiments performed, which gave similar results. The letters indicate different statistically significant groups (ANOVA, $P \leq 0.05$, Bonferroni test). B, Macroscopic disease symptoms of inoculated plants at 7 dpi. C and D, Fungal hyphae (C) and plant cell death (D) were visualized in the inoculated leaves by lactophenol TB 1 dpi. E, Hydrogen peroxide accumulation in inoculated leaves was determined by DAB staining at 1 dpi. F, Callose deposition in inoculated leaves (1 dpi) was visualized using aniline blue, and the number of total pixels of these depositions per μm^2 was quantified (G). At least four leaves from each genotype were used for pixel quantification in the two experiments performed that gave similar results. Bars = 10 μm.

(Fig. 2B; Supplemental Table S4). Thus, 67% (90 genes) of the genes constitutively up-regulated (134 genes) in *aba1-6* mutant plants were also induced in wild-type plants upon *PcBMM* infection (Fig. 2B) and might be considered as constitutively up-regulated defensive genes. Meta-analysis, using Genevestigator tools (Zimmermann et al., 2004), demonstrated that the expression of a high number of these up-regulated genes was also induced after Arabidopsis infection with other necrotrophic fungi such as *B. cinerea* (60 genes) and with other pathogens like *Phytophthora infestans* (70 genes), *Erysiphe orontii* (27 genes), *Erysiphe cichoracearum* (31 genes), or *P. syringae* (19 genes); in contrast, a reduced number of these genes were found to be differentially regulated after insect or nematode attack (eight and five genes, respectively). About 63% (57 genes) of the genes up-regulated both in *aba1-6* untreated plants and in *PcBMM*-inoculated wild-type plants were also up-regulated by JA, SA, or ET treatment. These data indicated that an extensive cross talk existed among these pathways and ABA signaling.

In addition, 33 (42%) out of the 79 probe sets down-regulated in untreated *aba1-6* were also down-regulated after infection of wild-type plants with *PcBMM*. Interestingly, expression of a high percentage of these genes was also down-regulated after infection with *B. cinerea* (67%) and *P. infestans* (48%), as revealed by meta-analysis (data not shown). The expression of 30% of these *aba1-6* and *PcBMM* down-regulated genes was found to be repressed either by SA or ET treatment, further supporting the existence of a negative interplay among these signaling pathways in response to pathogen infection.

Functional classification of the proteins encoded by the genes differentially regulated in untreated *aba1-6* mutant and in *PcBMM*-inoculated wild-type plants was performed using the Classification SuperViewer tool (Bio-Array Resource; http://bar.utoronto.ca/; Toufighi et al., 2005). Notably, the most overrepresented cellular component categories were "cell wall" ($P = 7 \times 10^{-15}$) and "extracellular" ($P = 3 \times 10^{-9}$), and the most overrepresented biological process categories were "response to stress" ($P = 2 \times 10^{-17}$) and "response to abiotic and biotic stimulus" ($P = 2 \times 10^{-12}$; Fig. 2C). Among the proteins in the "biotic stress" functional category, there were some previously described to be involved in defense against necrotrophs (Table I): (1) PDF1.2, PR-1, and PR-4, defensive markers of the JA/ET, SA, and ET pathways, respectively; (2) the respiratory burst oxidase homolog D (RbohD), an enzymatic subunit of the plant NADPH oxidase involved in the generation of hydrogen peroxide; (3) the transcription factors WRKY33 and ORA59; and (4) several proteins, such as CYP81F2, PAD3, and PEN3, involved in the synthesis and delivery of Trp-derived secondary

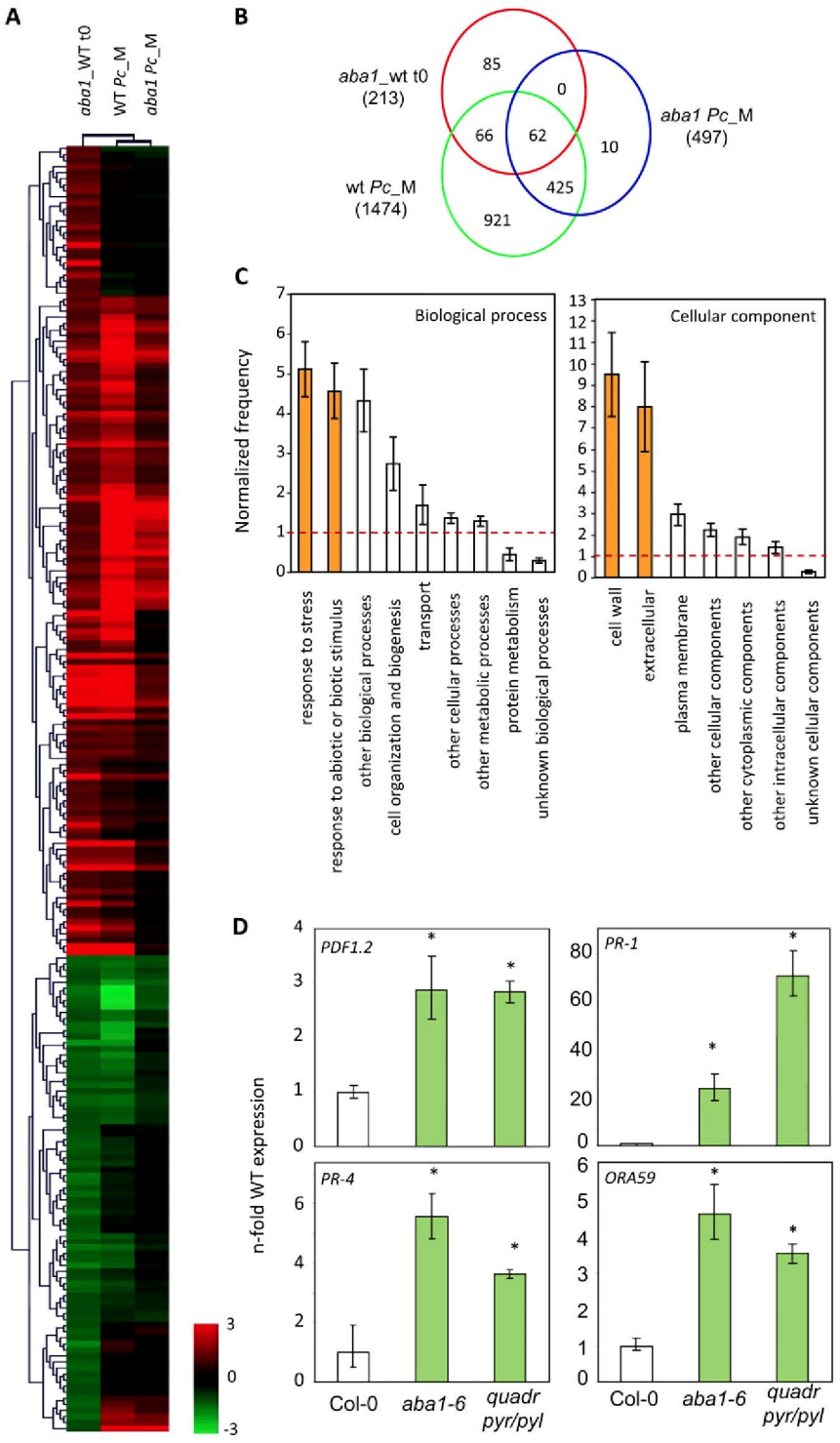

Figure 2. Transcriptomic profiles of untreated *aba1-6* mutant and *PcBMM*-inoculated wild-type plants strongly overlapped. A, Hierarchical clustering of the 213 differentially expressed probe sets in untreated *aba1-6* mutant plants compared with wild-type plants (*aba1*_WT t0). Genes (rows) and experiments (columns) were clustered with the Multiexperiment Viewer (The Institute for Genomic Research) using Pearson uncentered distance and average linkage. The columns corresponded to untreated *aba1-6* compared with wild-type plants (*aba1*_WT t0) and *PcBMM*-inoculated wild-type and *aba1-6* plants at 1 dpi compared with their respective mock samples (WT *Pc*_M and *aba1 Pc*_M, respectively). B, Venn diagram showing overlapping of the differentially regulated probe sets ($P \leq 0.01$, ANOVA, Benjamini and Hochberg multiple testing correction) in the untreated *aba1-6* mutant line (red circle), in *PcBMM*-inoculated wild-type plants (green circle), and in *PcBMM*-inoculated *aba1-6* plants (blue circle). The numbers of differentially regulated probe sets in each treatment and of common genes among these treatments are indicated. C, The 128 probe sets that were common to untreated *aba1-6* plants and *PcBMM*-inoculated wild-type plants were classified using tools of the Bio-Array Resources for Plant Biology (http://bar.utoronto.ca/). The normalized score value for each functional class is represented. Normalized score values greater than 1 indicate overrepresented classes in comparison with the whole genome. D, Validation of differentially expressed genes in *aba1*-6 mutants. The expression of *PDF1.2*, *PR-1*, *PR-4*, and *ORA59* genes was determined by qPCR in untreated wild-type plants (WT; Col-0), *aba1-6*, and *quadruple pyr1-pyl1pyl2pyl4* (*quadr pyr/pyl*) mutants. Values were normalized to Arabidopsis *UBIQUITIN21* expression levels and, for a better comparison with transcriptomic data, were represented as relative fold expression to the one in wild-type plants. Bars represent average values ± SD of two technical replicates. Asterisks indicate differences statistically significant from those of Col-0 plants ($P \leq 0.05$, ANOVA, LSD test). Data are from one out of three independent experiments, which gave similar results.

metabolites, like camalexin and indole glucosinolates. Similarly, some genes encoding cell wall-related proteins were found to be misregulated in the *aba1-6* mutant compared with wild-type plants (Supplemental Table S5). Remarkably, cell wall architecture/alteration has been recently linked to the regulation of tomato and Arabidopsis resistance to necrotrophic pathogens, such *B. cinerea* and *PcBMM*, respectively (Asselbergh et al., 2007; Sánchez-Rodríguez et al., 2009; Delgado-Cerezo et al., 2012).

To validate the transcriptomic data, the steady-state expression of some defensive genes (*PDF1.2*, *PR-1*, *PR-4*, and *ORA59*), which were up-regulated in *aba1-6* plants, was determined in untreated wild-type plants and the ABA-resistant mutants (*aba1-6* and *quadruple pyr/pyl*). qPCR analysis demonstrated that the expression of these

Table I. *Defense-related genes constitutively up-regulated in the aba1-6 mutant and induced in wild-type plants after PcBMM challenge*

Gene Description	Locus	Fold Change		Reference
		aba1/Wild Type[a]	Wild Type (*Pc*/M)[b]	
PR-4/HEL (hevein-like)	AT3G04720	3.43	3.16	Hejgaard et al. (1992); Potter et al. (1993)
PDR8 (pleiotropic drug resistance8)/PEN3	AT1G59870	1.83	1.07	Stein et al. (2006)
PR-1 (pathogenesis-related1)	AT2G14610	4.10	3.15	Van Loon and Van Strien (1999)
PDF1.2B (plant defensin1.2B)	AT2G26020	5.81	7.78	Thomma et al. (2002)
PLP2 (patatin-like protein)	AT2G26560	4.12	3.33	La Camera et al. (2005)
WRKY33	AT2G38470	3.74	1.15	Zheng et al. (2006)
ATTI1 (trypsin inhibitor protein1)	AT2G43510	2.44	1.36	Silverstein et al. (2005)
CHI-B; PR-3 (basic chitinase)	AT3G12500	4.13	3.39	Verburg and Huynh (1991)
PRX33 (peroxidase33)	AT3G49110	2.02	1.20	Bindschedler et al. (2006); O'Brien et al. (2012)
OSM34 (osmotin34)	AT4G11650	3.74	2.41	Monteiro et al. (2003)
Blue copper-binding protein	AT5G20230	4.03	3.05	Mishina and Zeier (2007)
PDF1.2A (plant defensin1.2A)	AT5G44420	6.20	8.90	Manners et al. (1998) Thomma et al. (2002)
RBOHD (respiratory burst oxidase protein D)	AT5G47910	2.26	1.56	Torres et al. (2005)
PAD3 (phytoalexin deficient3; CYP71B15)	AT3G26830	6.39	1.16	Glazebrook and Ausubel (1994); Zhou et al. (1999)
CYP81F2 (cytochrome P450)	AT5G57220	4.36	1.50	Bednarek et al. (2009); Clay et al. (2009)
ORA59 (octadecanoid-responsive Arabidopsis AP2/ERF59)	AT1G06160	3.20	2.88	Pré et al. (2008)
PNP-A (plant natriuretic peptide A)	AT2G18660	1.13	5.05	Meier et al. (2008)
ATGRXS13 (glutaredoxin13)	AT1G03850	1.55	3.25	La Camera et al. (2011)
WRKY26	AT5G07100	1.20	1.88	Eulgem and Somssich (2007)

[a]Normalized, average fold ratios ($\log_2$) of *aba1*-6 versus wild-type plants at time zero. [b]Normalized, average fold ratios ($\log_2$) of *PcBMM*-inoculated versus mock-treated wild-type plants. Genes were selected using values of $P \leq 0.01$ (see "Materials and Methods").

genes was higher in both ABA-deficient and ABA signaling mutants than in wild-type plants (Fig. 2D). The role of one of these differentially expressed genes in Arabidopsis resistance to *PcBMM* was validated by demonstrating the enhanced susceptibility to this fungus of the *wrky33-1* mutant, which is defective in the WRKY33 transcriptional factor previously shown to control resistance to the necrotroph *B. cinerea* (Supplemental Fig. S3; Zheng et al., 2006). These data further corroborated the relevance in plant immunity of constitutively up-regulated genes in the Arabidopsis *aba1-6* and *quadruple pyr/pyl* mutants.

Hormonal Regulation of the Genes Differentially Expressed in *aba1-6* Plants

To gather additional information on the processes regulated by genes differentially regulated in *aba1-6*, meta-analysis was performed on the transcriptomic data of the untreated *aba1-6* mutant (Supplemental Table S1), wild-type plants inoculated with *PcBMM* (Supplemental Table S2), and publicly available transcriptomic data of Arabidopsis wild-type plants treated with the hormones SA, 1-aminocyclopropane-1-carboxylic acid (ACC), an ET precursor, or methyl jasmonate (Goda et al., 2008). Using Pearson uncentered hierarchical clustering on genes differentially regulated in *aba1-6* compared with wild-type plants upon inoculation with *PcBMM*, we found that the transcriptomic profiles of the untreated *aba1-6* mutant and *PcBMM*-inoculated wild-type plants clustered together and separately from the transcriptomic profiles of plants treated with the different hormones (Fig. 3), indicating that the overall transcriptomic profile of the *aba1-6* mutant could not be linked specifically to any of these hormones. Pearson uncentered hierarchical clustering differentiated seven statistically significant gene clusters using a distance threshold of 0.7 (Fig. 3; data not shown). The largest clusters (I–IV) were further analyzed: clusters I to III corresponded to genes up-regulated in both untreated *aba1-6* and *PcBMM*-inoculated wild-type plants, whereas cluster IV contained genes down-regulated in both *PcBMM*-inoculated wild-type plants and untreated *aba1-6* plants (Supplemental Table S6).

The presence of conserved putative cis-regulatory elements was determined in the genes included in clusters I to IV with Transplanta software (http://bioinfogp.cnb.csic.es/transplanta_dev/). Subsequently, TOMTOM (http://meme.nbcr.net/meme/cgi-bin/tomtom.cgi; Gupta et al., 2007) and PLACE (for Plant cis-acting Regulatory DNA Elements; http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999) databases were used to identify the statistically overrepresented regulatory elements in the promoters of the genes from each cluster. We observed that cluster I contained genes (14 out of 23) predicted to be predominantly up-regulated by JA treatment, some of

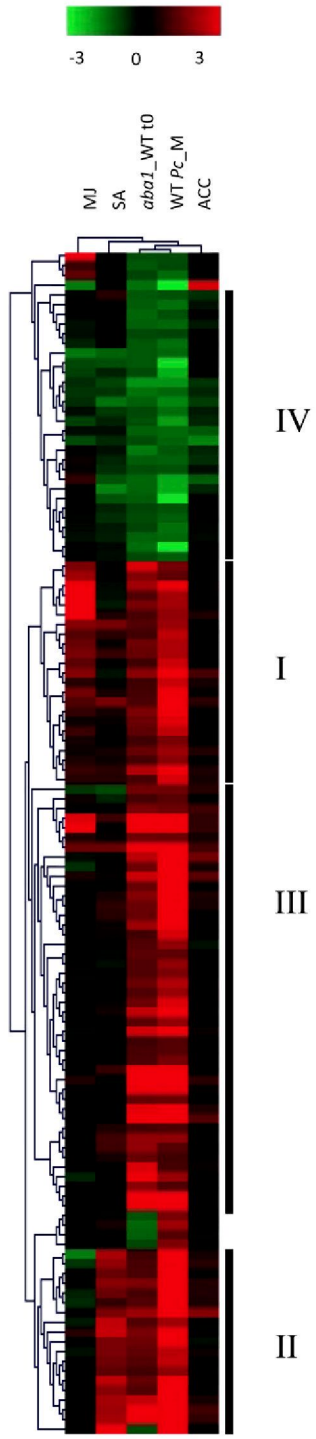

Figure 3. Hierarchical clustering of genes differentially regulated in untreated *aba1-6* and in *PcBMM*-inoculated wild-type plants. Hierarchical clustering is shown for the 128 probe sets differentially expressed in both *aba1-6* untreated plants (*aba1*_WT t0) and in *PcBMM*-inoculated wild-type plants (WT *Pc*_M) and those from treatments of Arabidopsis with different hormones (SA, ACC, and methyl jasmonate [MJ]; http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). All data sets were normalized with the robust multiarray average method using GeneSpring software. Genes (rows) and samples (columns) were clustered with the Multiexperiment Viewer (The Institute for Genomic Research) using Pearson uncentered distance and average linkage (threshold = 0.7). The resulting clusters (I–IV), which are indicated by vertical bars, were further analyzed, and the results are shown in Supplemental Table S5.

which encoded proteins (RbohD, PEN3, ATKTI1, and ATTI1) involved in plant basal resistance (Supplemental Table S6). Notably, the cis-regulatory elements of these JA-dependent genes were enriched in W-box sequences, suggesting a transcriptional regulation by WRKY transcription factors (Rushton et al., 1996). Cluster II contained genes (18 out of 19) associated with SA-mediated induction, such as defense-related genes like *PR-1*, *WRKY33*, *PNP-A* (encoding the plant natriuretic peptide A), *ATGRXS13*, and a *Receptor-Like Kinase* (Table I). Among these genes there were no cis-regulatory elements statistically overrepresented. The 44 genes in cluster III, which were not predominantly regulated by any of the hormones analyzed, encoded proteins involved in biotic and abiotic stress responses, such as CHIB, PR-4, PRX33, and PRX34 (Table I), cell wall-related proteins, and two transcription factors (WRKY26 and ORA59). The W-box cis-regulatory element was also overrepresented in the promoters of cluster III genes. Finally, nearly all the genes down-regulated in untreated *aba1-6* plants were contained in cluster IV, and they were found to be negatively regulated by either ACC or SA treatment and, to a lesser extent, by the JA signaling pathway. Among the genes found in this cluster, several encoded cell wall-related proteins (Fig. 3; Supplemental Table S6). There were no cis-regulatory elements statistically overrepresented in cluster IV genes.

The cluster analyses suggested that a significant number of the genes differentially regulated in *aba1-6* plants responded to different hormones, in particular to SA and JA. Moreover, a significant number of the differentially expressed genes in *PcBMM*-inoculated wild-type plants corresponded to genes that are up-regulated (110 probe sets) or down-regulated (87 probe sets) by ABA. Accordingly, we measured the concentration of these hormones in ABA-deficient and ABA signaling mutants and in wild-type plants to evaluate if the misregulation of these genes could be linked to alterations in hormone levels. SA content was not altered in untreated *aba1-6* and *quadruple pyr/pyl*, while it was lower in the *triple abi2-2* mutant plants (Supplemental Fig. S4A). The concentration of JA was lower in the three mutants than in wild-type plants (Supplemental Fig. S4A). As expected, the *aba1-6* mutant displayed lower ABA content than wild-type plants and *quadruple pyr/pyl* and *triple abi2-2* mutants, which are not impaired in ABA biosynthesis (Supplemental Fig. S4A). Inoculation of wild-type plants with *PcBMM* (1 dpi) induced an increase in the concentration of JA and SA (Supplemental Fig. S4B), which was consistent with the previously described positive roles of SA and JA in the regulation of Arabidopsis resistance to this fungal pathogen (Berrocal-Lobo et al., 2002). Interestingly, the

elevation of JA and SA contents in the *PcBMM*-inoculated ABA mutants did not exceed that in wild-type plants (Supplemental Fig. S4B). Notably, significant differences in the levels of ABA were not found among *PcBMM*-inoculated and mock-treated wild-type plants (Supplemental Fig. S4B).

SA, JA, and ET Signaling Pathways Are Essential for *aba1*-Mediated Resistance to *PcBMM*

To further determine if the enhanced resistance of *aba1-6* plants to *PcBMM* was dependent on JA, SA, and ET signaling, we genetically disrupted these pathways in the *aba1-6* mutant by generating the *sid2-1 aba1-6*, *ein2-1 aba1-6*, *aos aba1-6*, and *coi1-1 aba1-6* double mutants (Fig. 4; data not shown), which were impaired, respectively, in SA biosynthesis, ET signaling, and JA biosynthesis and signaling. All these double mutants were viable and showed developmental phenotypes that in some cases were a combination of those observed in their corresponding parental lines (Supplemental Fig. S5). Susceptibility of these double mutants to *PcBMM* was analyzed by spraying them alongside their parental lines and wild-type plants with a spore suspension (4×10^6 spores mL^{-1}) of the fungus. Notably, fungal biomass and DR were significantly higher in *sid2-1 aba1-6*, *ein2-1 aba1-6*, *aos aba1-6*, and *coi1-1 aba1-6* double mutants than in the *aba1-6* single mutant (Fig. 4, A and B; data not shown), indicating that the SA, ET, and JA signaling pathways are essential for the enhanced resistance of *aba1-6* to the necrotrophic fungus *PcBMM*. However, none of the double mutants tested was as susceptible as their corresponding parental lines *sid2-1*, *ein2-1*, or *aos* (Fig. 4, A and B). These data indicate that all these signaling pathways partially contributed to *aba1*-mediated resistance, as any of them on its own was sufficient to explain the enhanced resistance phenotype of *aba1-6*.

The impact of hormone imbalance in the resistance phenotype observed in the *sid2-1 aba1-6*, *ein2-1 aba1-6*, and *aos aba1-6* double mutants was studied by quantifying the expression levels of *PR-1*, *PR-4*, *LOX2*, and *PDF1.2* (marker genes of SA, ET, JA, and ET/JA signaling, respectively). The steady-state expression of *PR-1*, which was higher in the *aba1-6* mutant than in wild-type plants (Fig. 2D), was further enhanced in the *ein2-1 aba1-6* and *aos aba1-6* double mutants (Fig. 4C). As expected, *PR-1* expression was not detected in the *sid2-1* and *sid2-1 aba1*-6 mutants impaired in SA biosynthesis (Fig. 4C). These data suggested a negative regulation of *PR-1* expression by ABA, ET, and JA signaling pathways. The enhanced expression of *PR-4* in the *aba1-6* mutant was disrupted in the *ein2-1 aba1-6* plants but not in the *sid2-1 aba1-6* and *aos aba1-6* double mutants, further corroborating the ET-dependent regulation of the *PR-4* gene in the *aba1-6* background (Fig. 4C). No significant changes were observed in the expression levels of *LOX2*, a marker gene for the JA signaling pathways, in all the genotypes tested (Fig. 4C). The enhanced expression of the *PDF1.2* gene in the *aba1-6* mutant (Figs. 2D and 4C) was partially blocked in the *sid2-1 aba1-6* and *ein2-1 aba1-6* double mutants, whereas it was not affected in the *aos aba1-6* genotype (Fig. 4C). All together, these data illustrate the complex interplay between hormone signaling pathways in the regulation of defensive genes in Arabidopsis resistance responses.

JA, SA, and ET Signaling Pathways Do Not Interfere with ABA Function in the Regulation of Arabidopsis Resistance to Abiotic Stresses

ABA signaling also has a role in the regulation of plant responses to abiotic stress, such as drought and osmotic stress. Thus, the *aba1* mutant was shown to be impaired in ABA-mediated resistance to desiccation (Xiong et al., 2002). Taking advantage of the double mutants generated in this work, we decided to assess whether the SA, ET, and JA signaling pathways might interfere with the regulatory function of ABA signaling in abiotic stresses. We determined plant water loss (represented as percentage of lost weight) at different time points (0–3 h) in wild-type plants and the single and double mutants. As shown in Figure 5, water loss was similar in Col-0, *sid2-1*, *ein2-1*, and *aos* genotypes, which harbored the wild-type *ABA1* allele. Interestingly, the *sid2-1 aba1-6*, *ein2-1 aba1-6*, and *aos aba1-6* double mutants were as sensitive to this abiotic stress as the single mutant *aba1-6*, which showed an enhanced and faster water loss than that determined in Col-0 plants (Fig. 5). These data indicated that ET, SA, and JA signaling did not play a major role in the regulation of Arabidopsis responses to this abiotic stress and that they did not interfere with ABA function in the regulation of this process.

ABA Modulates Arabidopsis Cell Wall Composition

Arabidopsis resistance to necrotrophic fungi, such as *PcBMM*, can be regulated by remodeling plant cell wall structure and composition (Hernández-Blanco et al., 2007; Cantu et al., 2008; Sánchez-Rodríguez et al., 2009; Ramírez et al., 2011; Delgado-Cerezo et al., 2012). Ontology analyses of the genes differentially regulated in untreated *aba1-6* plants identified cell wall-related genes misregulated in the mutant compared with wild-type plants ($P = 7 \times 10^{-15}$; Supplemental Table S5). Thus, we determined cell wall structure/composition of the *aba1-6* mutant and compared it with that of wild-type plants. Cell walls from non-inoculated leaves of 3-week-old *aba1-6* and Col-0 plants were subjected to Fourier transform infrared (FTIR) spectroscopy to obtain qualitative spectratypes (i.e. cell wall phenotypes). The comparison of averaged FTIR spectra ($n = 15$) obtained between *aba1-6* and Col-0 showed significant differences (Fig. 6A). Much of the total sample variation (86%) was explained by principal component 1 (Fig. 6B). Wave numbers at 1,056, 1,080, and 1,109 cm^{-1}, which are common in the fingerprint for several cell wall polysaccharides (Kauráková et al.,

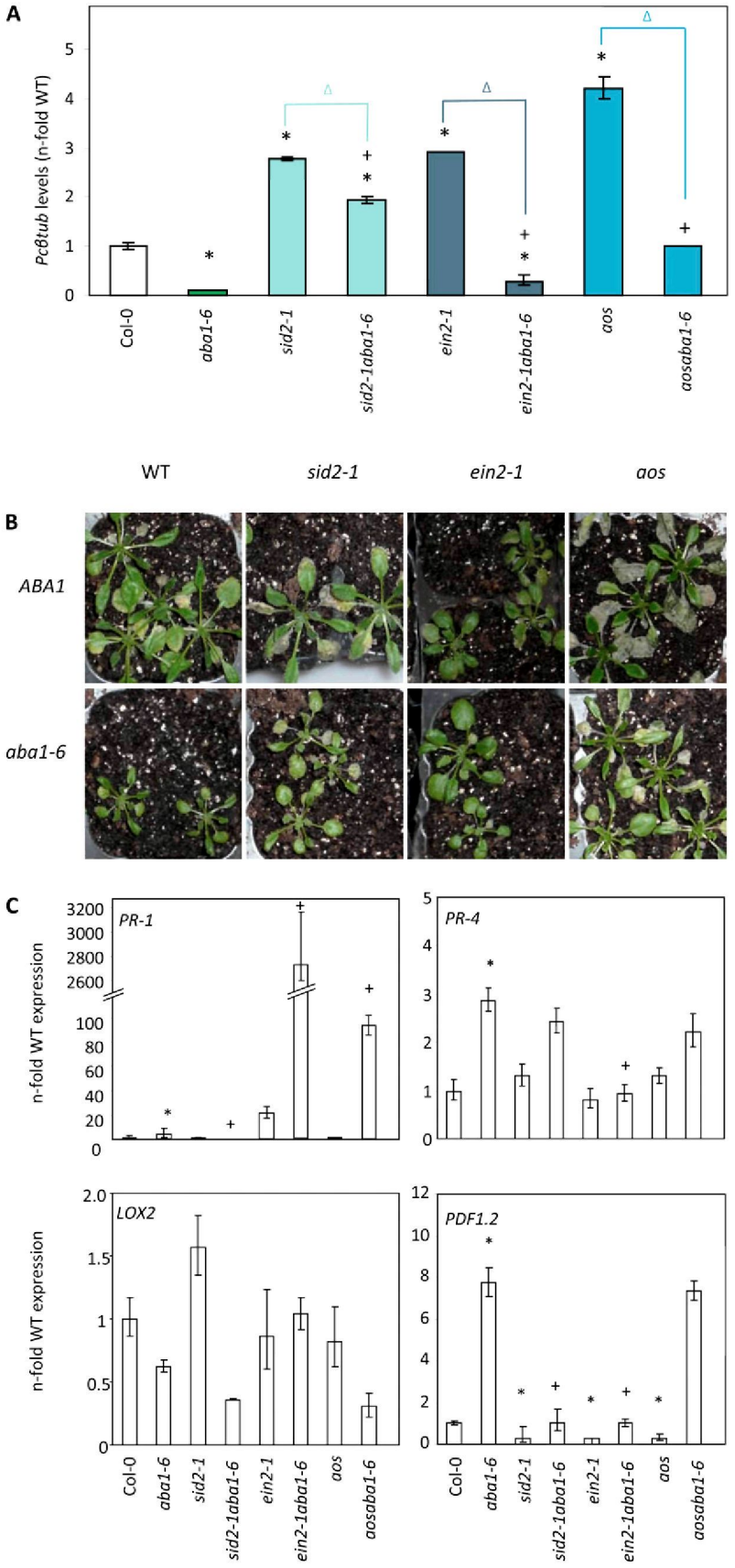

Figure 4. SA, ET, and JA are essential for *aba1*-mediated resistance to *PcBMM*. Wild-type plants (WT; Col-0), *aba1-6*, *sid2-1*, *ein2-1*, and *aos* single mutants, and *sid2-1 aba1*, *ein2-1 aba1-6*, and *aos aba1-6* double mutants were inoculated with a spore suspension (4×10^6 spores mL^{-1}) of *PcBMM*. A, Fungal biomass in the inoculated plants (3 dpi) was determined by qPCR using specific primers for *P. cucumerina* β-*Tubulin*. Values were normalized to Arabidopsis *UBIQUITIN21* expression levels and represented as fold increased expression compared with wild-type plants. Data represent average values of two technical replicates ± SE from one of the three experiments performed, which gave similar results. Asterisks indicate genotypes that show significant differences in fungal biomass compared with that in the wild-type; plus signs indicate double mutants that show significant differences in fungal biomass compared with that in *aba1-6* plants; and triangles indicate statistical differences in fungal biomass between the double mutants and the corresponding parental line ($P \leq 0.05$, ANOVA, Bonferroni test). B, Macroscopic disease symptoms of *PcBMM*-inoculated plants at 7 dpi. C, Expression of *PR-1* (*AT2G14610*), *PR-4* (*AT3G04720*), *LOX2* (*AT3G45140*), and *PDF1.2* (*AT5G44420*), marker genes of the SA, ET, JA, and ET+JA pathways, respectively, was determined by qPCR on untreated 3-week-old plants. Values are represented as fold increased expression compared with the wild-type. Data correspond to average values ± SD of two replicates from one representative experiment out of the three performed. Asterisks indicate single mutants showing a significant differential expression of the gene compared with the wild-type, whereas plus signs indicate double mutants showing significant differential expression of the gene compared with *aba1-6* plants ($P \leq 0.05$, ANOVA, LSD test).

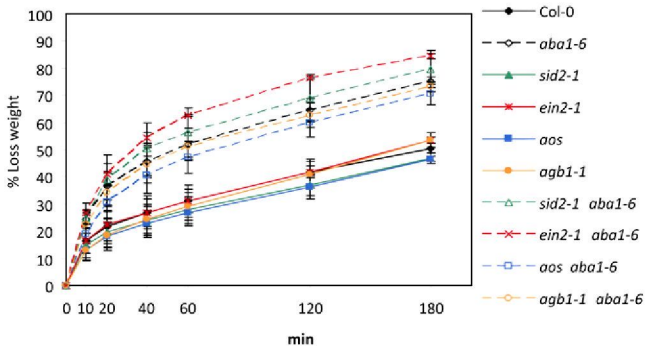

Figure 5. SA, ET, and JA pathways do not affect the transpiration rates of *aba1-6* plants. The sensitivity of wild-type plants (Col-0), *aba1-6*, *sid2-1*, *ein2-1*, and *aos* single mutants, and *sid2-1 aba1*, *ein2-1 aba1-6*, and *aos aba1-6* double mutants to water desiccation is shown. Transpiration rates were determined as percentage lost weight at different time points (0–180 min). Each data point represents the mean of three replicates ± SE. This experiment was repeated three times and gave similar results.

2000), and 1,544 and 1,654 cm^{-1}, which are associated with proteins, were relatively more abundant in Col-0 than in *aba1-6* cell walls (Fig. 6C). On the contrary, bands at 825, 889, and 935 cm^{-1} were more intense in *aba1-6* than in wild-type plants (Fig. 6C). Due to the complexity of infrared spectra even after principal component analysis, it was difficult to assign infrared band differences to specific cell wall polysaccharides.

To obtain a more quantitative analysis of the cell wall changes in *aba1-6* plants, we determined the noncellulosic neutral monosaccharide composition of leaves from noninoculated 3-week-old *aba1-6* and Col-0 plants (Blakeney et al., 1983; Reiter et al., 1993) as well as cellulose and uronic acid contents of their walls (Fig. 6, D and E). We found that the amount of cellulose was much lower in *aba1-6* samples than in wild-type plants, whereas the content of uronic acid was much higher in the mutant than in the wild-type plants (Fig. 6D). No significant changes were observed in noncellulosic neutral sugar between *aba1-6* samples and wild-type plants (Fig. 6E). To further correlate these cell wall alterations in the *aba1-6* mutant with its resistance phenotype, we determined the cellulose and uronic acid contents, as well as the noncellulosic neutral monosaccharide composition, in leaves from noninoculated 3-week-old *quadruple pyr/pyl* and *triple abi2-2* mutants. As shown in Supplemental Figure S6, no significant alterations in the contents of uronic acid and cellulose were found between these mutants and wild-type plants, whereas a subtle reduction in the content of noncellulosic neutral sugars was found in the *triple abi2-2* mutant compared with that in wild-type plants. All together, these results revealed that disruption of ABA biosynthesis, but not ABA signaling, leads to a modification of cell wall structure and composition. However, the lack of correlation between altered cell wall chemistry and basal resistance in the *pyr/pyl* mutant and the triple *abi2-1* mutant suggests that these cell wall modifications have no significant contribution to ABA-regulated resistance.

DISCUSSION

ABA has multiple and diverse functions in plants, such as the regulation of development-associated processes, the response to abiotic stresses, and the outcome of pathogen infections (Fujita et al., 2006; Wasilewska et al., 2008). For the latter, positive and negative functions have been described for ABA depending on the specific plant-pathogen interaction studied (Mauch-Mani and Mauch, 2005; Ton et al., 2009). Here, we show that ABA plays a negative regulatory function in balancing Arabidopsis resistance response to *PcBMM*, a necrotrophic fungal pathogen that naturally colonizes a broad range of Arabidopsis accessions (Llorente et al., 2005; Sánchez-Vallet et al., 2010). The *aba1-6* mutant defective in ABA biosynthesis and the *quadruple pyr/pyl* mutant impaired in four ABA receptors (Niyogi et al., 1998; Park et al., 2009) were more resistant to *PcBMM* than wild-type plants, whereas the triple *hab1-1abi1-2abi2-1* mutant, which has a constitutive activation of ABA signaling due to the impairment of three PP2C phosphatases (from clade A) that negatively regulate ABA signaling (Rubio et al., 2009), displayed an enhanced susceptibility to this fungal pathogen (Fig. 1). Thus, in addition to ABA biosynthesis, we show here that the ABA signaling pathway is essential for the suppression of Arabidopsis resistance to necrotrophic fungi, as impairment or constitutive activation of the pathway led to enhanced or reduced resistance responses, respectively. In line with these data, treatment of the ABA-deficient mutant *aba1-1* (Landsberg *erecta* background) with ABA (50 μM) reverted its enhanced resistance to *PcBMM* to the susceptibility levels observed in wild-type plants (Supplemental Table S7). The data presented here are consistent with the negative regulatory role of ABA seen on resistance in tomato to the necrotrophic pathogens *B. cinerea* and *D. dadantii* and in Arabidopsis to the fungi *F. oxysporum* and *B. cinerea* (Audenaert et al., 2002; Anderson et al., 2004; Asselbergh et al., 2008a; L'Haridon et al., 2011). In contrast, ABA plays a positive regulatory role in Arabidopsis basal resistance to the necrotrophic fungus *A. brassicicola* or the vascular oomycete *P. irregulare* and in the modulation of *ocp3* resistance to necrotrophic fungi (Adie et al., 2007; Flors et al., 2008; García-Andrade et al., 2011). Strikingly, a positive role of ABA in resistance to *P. cucumerina* has been described previously (e.g. the *aba2* mutant was shown to be hypersusceptible to the fungus), and this function has been associated with the regulation of callose deposition upon infection (García-Andrade et al., 2011). However, in these experiments, the plants used for inoculation were older (5 weeks old) than those used here (3 weeks old), and drop inoculation instead of spray inoculation of expanded leaves was performed (García-Andrade et al., 2011). Moreover, under our

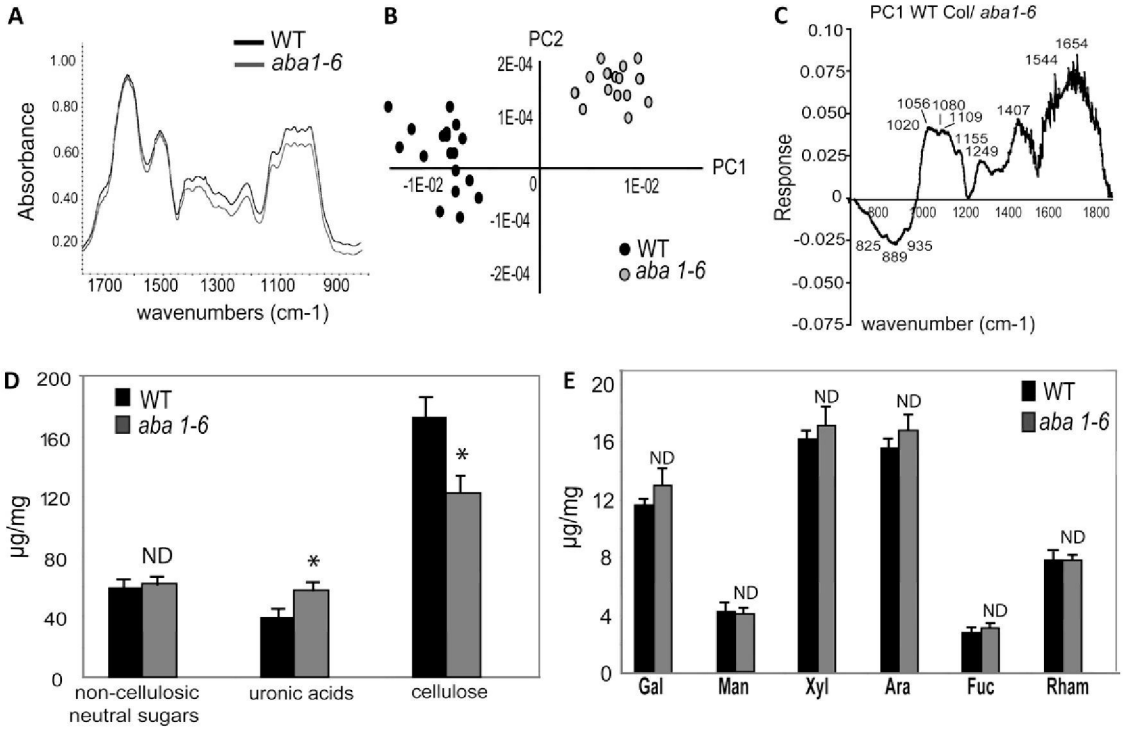

Figure 6. FTIR and biochemical analyses of the cell walls from *aba1-6* mutant and wild-type (WT) plants. A, FTIR average spectra (n = 15) from 3-week-old rosettes of Col-0 wild-type plants and the *aba1-6* mutant. B, Biplot analysis showing a clear separation of *aba1-6* and wild-type plants. C, Mid-infrared spectra were analyzed by the covariance-matrix approach for principal component 1 (PC1). D and E, Quantification of cellulose, total uronic acid content (g mg^{-1} dry weight; D), and individual neutral sugars (mol %) from the noncellulosic carbohydrate fraction (E) from the cell walls of wild-type plants (black bars) and the *aba1-6* mutant (gray bars). Data represent average values ± SE of three replicates. Asterisks indicate values statistically different from those of mutant plants (Student's t test); ND indicates no significant differences ($P \leq 0.05$).

experimental conditions, callose deposition does not seem to be essential for resistance to *PcBMM*, as the susceptibility of the callose-deficient *pmr4* plants to the fungus was similar to that of wild-type plants (Supplemental Fig. S2). The data presented here reinforce the different roles that ABA plays in plant basal resistance depending on the plant-pathogen interaction analyzed and also suggest a putative effect of plant developmental stage and growth environment conditions on ABA regulatory function. This last conclusion is in line with the recently described effect of humidity on the effectiveness of Arabidopsis wound-induced resistance to *B. cinerea* (L'Haridon et al., 2011).

Comparative transcriptomic analysis of the *aba1-6* mutant and wild-type plants demonstrated that in *aba1-6* there is a constitutive activation of resistance responses, as the expression of a significant subset of defense-related genes was up-regulated in the mutant compared with wild-type plants (Table I). Furthermore, we found a significant overlap (60% of the genes) between the transcriptomic profiles of untreated *aba1-6* mutant and *P. cucumerina*-infected wild-type plants 1 dpi. This is a clear indication that the Arabidopsis defensive responses activated upon fungal infection were constitutively up-regulated in *aba1-6* plants and, therefore, that ABA negatively regulates these responses against this fungal pathogen. Among the defensive proteins encoded by constitutively up-regulated genes in *aba1-6* were antimicrobial proteins (e.g. PDF1.2, PR-1, and LTP3), enzymes involved in the biosynthesis and target delivery of Trp-derived secondary metabolites, such as indole glucosinolates and camalexin (e.g. PEN3, PAD3, and CYP81F2), and the ATRBOHD and PRX33/PRX34 proteins that are involved in ROS generation (Fig. 2). The enhanced accumulation of antimicrobial proteins and secondary metabolites in Arabidopsis has been shown to confer broad-spectrum resistance to pathogens, including the necrotroph *PcBMM* (García-Olmedo et al., 2001; Stein et al., 2006; Hernández-Blanco et al., 2007; Bednarek et al., 2009; Sánchez-Vallet et al., 2010). ROS accumulation has been linked to the enhanced resistance phenotype of some ABA mutants (Asselbergh et al., 2007; L'Haridon et al., 2011), but a significant increase in ROS production upon *PcBMM* infection was not observed in the ABA mutants in comparison with wild-type plants (Fig. 1E). Notably, some transcriptional factors, such as WRKY33 and ORA59, that regulate Arabidopsis immune responses to pathogens, including necrotrophic fungi (Supplemental Fig. S3; Zheng et al., 2006; Pré et al., 2008), were also up-regulated in unchallenged *aba1-6* and *quadruple pyr/pyl* mutants (Fig. 2; data not shown). Interestingly, the

cis-regulatory elements of the *aba1-6* up-regulated genes were enriched in W-box and GCC-box sequences that bind transcriptional factors such as WRKY33 and ORA59, respectively (Rushton et al., 1996; Zarei et al., 2011). These data further corroborate the link between ABA signaling and the Arabidopsis resistance response to the necrotrophic fungus *PcBMM*.

Hierarchical clustering of genes differentially regulated in the *aba1-6* mutant identified four statistically significant clusters (I–IV; Fig. 3; Supplemental Table S6). The functions of the genes contained in these clusters suggested an extensive and complex cross talk among different signaling pathways. For example, two of the *aba1-6* clusters contained up-regulated genes whose expression was positively modulated by either JA or SA treatment (Fig. 3). Genetic disruption of these pathways by generating the *coi1-1 aba1-6*, *aos aba1-6*, and *sid2-1 aba1-6* double mutants led to a partial impairment of the *aba1-6* resistance phenotype (Fig. 4; data not shown). Similarly, the cluster analysis suggested an ET function in *aba1-6* resistance, which was further corroborated by showing that the *ein2-1 aba1-6* double mutant was more susceptible to *PcBMM* than *aba1-6* plants (Fig. 4). These data are in accordance with previous reports describing the antagonistic interaction between ABA and the JA, ET, and SA signaling pathways in the regulation of plant resistance to pathogens. With a few exceptions, these antagonistic interactions were hypothesized based on comparative gene expression analysis (Anderson et al., 2004; Adie et al., 2007; de Torres-Zabala et al., 2007; Asselbergh et al., 2008b; Flors et al., 2008). Here, we have genetically validated some of the interactions of this complex network and also found additional, previously uncharacterized ones (Fig. 7). Notably, the interaction between ABA and the JA and SA hormones in balancing the resistance response of *aba1-6* to *PcBMM* seems to take place at the signaling level, as the content of these hormones in *aba1-6* and *quadruple pyr/pyl* mutants (untreated or *PcBMM* inoculated) was not higher than those found in wild-type plants. Moreover, ABA content was not increased in *PcBMM*-inoculated wild-type plants (Supplemental Fig. S4), and exogenous application of ABA (50 μM) did not have any effect on the resistance of wild-type plants, whereas it restored the resistance of the *aba1* mutant to the susceptible levels of wild-type plants (Supplemental Table S7). In contrast to their genetic interaction in the regulation of Arabidopsis innate immunity, JA, ET, and SA signaling do not interfere with ABA function in the regulation of Arabidopsis responses to abiotic stress, such as drought (Fig. 5).

The intermediate resistance phenotype observed in the double mutants in comparison with that of *aba1-6* plants suggests that ABA, JA, ET, and SA signaling pathways cooperatively contribute to Arabidopsis resistance to *PcBMM*. How these pathways interact at the molecular level to regulate this resistance needs further characterization, but it seems to be more complex than initially anticipated (Tsuda et al., 2009). For example, the constitutive overexpression of the SA marker gene *PR-1* in the *ein2-1 aba1-6* and *aos aba1-6* double mutants (Fig. 4C) was not linked to an enhanced accumulation of SA in these double mutants (data not shown). Moreover, the ET/JA marker gene *PDF1.2* was similarly up-regulated in the *aba1-6* plants and in the *aos aba1-6* double mutant defective in JA biosynthesis (Fig. 4C). Therefore, the results presented here indicate that an intricate and complex regulatory network among these pathways occurs upon Arabidopsis challenge with necrotrophic pathogens (Fig. 7). It cannot be discarded that additional hormones such as auxin, gibberellin, cytokinin, and brassinosteroids might be involved in this multifaceted regulatory network conferring resistance to necrotrophic fungi (Bari and Jones, 2009).

Comparative transcriptomic analyses and genetic data revealed that in addition to the defensive responses regulated by hormones, other mechanisms might contribute to explain the resistance phenotypes of ABA mutants. For example, a significant number of the *aba1-6* differently regulated genes encoded proteins that were related to cell wall biosynthesis/modification (Fig. 2C). In line with these data, we found that *aba1-6* plants, which have a dwarf phenotype, showed alterations in the structure/composition of their walls compared with that of wild-type plants (Fig. 6), further suggesting that *aba1-6* resistance can be partially dependent on these wall alterations. Our data are in line with the

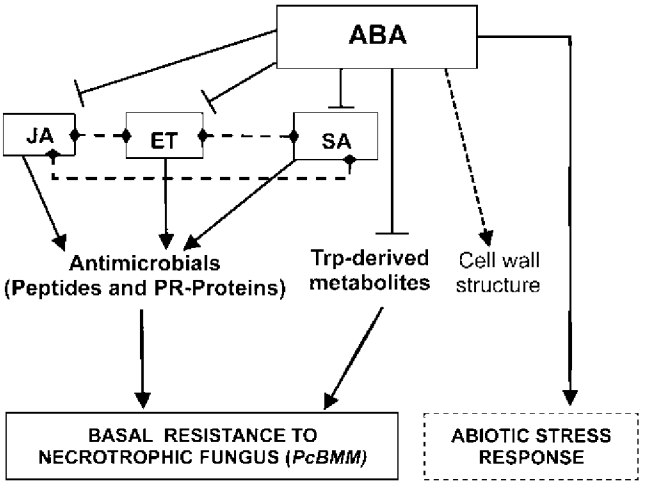

Figure 7. The complex regulatory function of ABA in Arabidopsis resistance to the necrotrophic fungus *PcBMM*. Upon Arabidopsis infection with the fungus, the activation of ET, SA, and JA pathways and the biosynthesis of Trp-derived metabolites occur, positively regulating (arrows) the basal resistance response. ABA signaling negatively regulates (lines) these pathways; therefore, inactivation of ABA signaling (e.g. *aba1-6* or *quadruple pyr/pyl*) leads to their constitutive activation and an enhanced resistance to the fungus. Previously described interactions among ET, SA, and JA signaling are indicated by dotted lines (for additional details, see text). The putative function of ABA in regulating wall architecture is indicated by the dotted arrow. The regulation of abiotic stress responses by ABA seems to be independent of SA, ET, and JA.

described cell wall alterations found in the tomato ABA-deficient *sitiens* mutant (Asselbergh et al., 2007) and with the hypothesized modulation of cell wall architecture by ABA signaling (Gimeno-Gilles et al., 2009). The relevance of the cell wall in directly mediating the resistance of Arabidopsis to different pathogens, including *PcBMM*, has been reported previously (Hernández-Blanco et al., 2007; Denoux et al., 2008; Sánchez-Rodríguez et al., 2009; Ramírez et al., 2011; Delgado-Cerezo et al., 2012). However, we did not find similar wall modifications in the *PcBMM*-resistant *quadruple pyr/pyl* mutant; therefore, it is tentative to speculate about the putative contribution of *aba1-6* wall alterations to *PcBMM* resistance. Also, a decrease in cuticle permeability and the alteration in callose deposition at the cell wall have been suggested to explain the resistance phenotypes of Arabidopsis and tomato ABA-deficient mutants (Ton and Mauch-Mani, 2004; Asselbergh et al., 2007a; García-Andrade et al., 2011; L'Haridon et al., 2011). However, in our plant growth and inoculation conditions, callose does not seem to contribute to the enhanced resistance to *PcBMM* of the *aba1-6* and *quadruple pyr/pyl* mutants (Fig. 1E; Supplemental Fig. S2). In conclusion, with this work, we elucidate the complex role that phytohormones, in particular ABA, play in balancing plant resistance to pathogens.

MATERIALS AND METHODS

Biological Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown in phytochambers on a sterilized mixture of soil and vermiculite (3:1) with a 10-h-day/14-h-night photoperiod, a temperature of 22°C day/20°C night, and 50% relative humidity (Hernández-Blanco et al., 2007). Light intensity was fixed to 120 to 150 μmol m^{-2} s^{-1}, according to Weigel and Glazebrook (2002). The following lines, in the Col-0 background, were used: *aba1-6* (Niyogi et al., 1998), *pyr1-pyl1pyl2pyl4* (Park et al., 2009), *hab1-1abi1-2abi2-2* (Rubio et al., 2009), *sid2-1* (Nawrath and Métraux, 1999), *ein2-1* (Guzmán and Ecker, 1990), *agb1-1* (Lease et al., 2001), *coi1-1* (Xie et al., 1998), *wrky33* (*salk_006603*), and *pmr4* and *pmr4 sid2-1* (Nishimura et al., 2003). The *aos* mutant is in the Col-6 background (*gl1*; Park et al., 2002), and the *aba1-1* allele is in the Landsberg *erecta* background (Koornneef et al., 1982). The *Plectosphaerella cucumerina* strain *BMM* was provided by Dr. B. Mauch-Mani (University of Neuchatel).

Double mutants were generated by standard genetic crosses followed by identification of the mutant alleles. Genotyping of the *aba1-6* mutation was confirmed by sequencing the PCR product of 5′-GAATCGGAGGATTGGTGTTTG-3′ and 5′-CTCCAGAATCTTCAAAATCAAC-3′. The *aos* mutation was confirmed by detection of the T-DNA insert using the flanking primers (5′-GTACGATCAAAGCCGTTGAAATTCCGAGTTTT-3′ and 5′-TGGAACAAGAAAACAGATGGACTACACAGGT-3′) and the T-DNA internal primer (5′-CGGGCCTAACTTTTGGTGTGATGATGCT-3′; Park et al., 2002). The *sid2-1* mutation was detected by selective digestion with *MunI* of the product of 5′-TGTCTGCAGTGAAGCTTTGG-3′ and 5′-CACAAACAGCTGGAGTTGGA-3′. The *ein2-1* mutation was selected as reported (Guzmán and Ecker, 1990). The *coi1-1* mutant was selected by determining plant insensitivity to JA (Xie et al., 1998).

Biological Assays

Three-week-old Arabidopsis plants were spray inoculated with a spore suspension (4×10^6 spores mL^{-1}) of *PcBMM* (0.6 mL per pot containing five plants). After inoculation, plants were transferred to a growth chamber with a 10-h-day/14-h-night photoperiod, a temperature of 24°C day/22°C night, and light intensity was maintained at 120 to 150 μmol m^{-2} s^{-1}. Inoculated plants were kept under high humidity in covered trays. Disease progression was estimated by determining the average DR (0–5; 0, no infection; 5, dead plant), TB staining, and relative quantification of fungal DNA by qPCR as described previously (Sánchez-Vallet et al., 2010). At least three independent experiments were performed, and statistically significant differences among the inoculated Arabidopsis genotypes were determined by one-way ANOVA and Bonferroni post hoc test, as reported previously (Sánchez-Rodríguez et al., 2009). For ABA treatment, *aba1-1* and wild-type plants were sprayed with ABA (50 μm) 24 and 48 h prior to inoculation with *PcBMM*, and then the DR was determined at different dpi and expressed as a percentage of disease susceptibility in wild-type plants (100%). Water loss assays were performed as described by Saez et al. (2006). Three samples containing four rosettes of 3-week-old plants were excised and weighed separately at different time points, and the average values (±SE) were determined. This experiment was performed three times.

Plant Tissue Staining

TB staining was performed on mock- and *PcBMM*-inoculated leaves at 20 hpi (for hyphal growth analysis) and 48 hpi (for cell death detection; Sánchez-Vallet et al., 2010). To determine the production of hydrogen peroxide, DAB (Sigma) staining was performed on at least six leaves collected 24 hpi and vacuum infiltrated with the DAB solution (1 mg mL^{-1}), placed in a plastic box under high humidity until brown precipitate was observed (3 h), and then fixed with 3:1:1 ethanol:lactic acid:glycerol (Torres et al., 2002). Callose deposition was determined by aniline blue staining in leaves harvested 24 hpi, which were incubated in 95% ethanol and then washed with a solution of 0.07 M phosphate buffer (pH 7) prior to their staining with aniline blue (0.02% in 0.07 M phosphate buffer, pH 7; Luna et al., 2011). Leaves were mounted on slides with glycerol and observed with an epifluorescence microscope using UV filters. Pixel quantification of callose was performed using Adobe Photoshop (Luna et al., 2011). At least four leaves per genotype were used for pixel quantification in the experiments performed.

Gene Expression Analyses

RNA extractions from *PcBMM*-inoculated or mock-treated plants were done as described (Llorente et al., 2005). Real-time qPCR analyses were performed as reported previously (Sánchez-Vallet et al., 2010). The oligonucleotide sequences, designed using Primer Express version 2.0 (Applied Biosystems), used for qPCR have been described previously (Sánchez-Vallet et al., 2010), except those of *ORA59* (5′-GCAGCCTCGCAGTACTCAATTT-3′ and 5′-TCAAGGCTATCACCGGAGACTC-3′) and *LOX2* (5′-ATCAACAAGCCCCAATGGAA-3′ and 5′-CGGCGTCATGAGAGATAGCAT-3′). qPCR results are mean values (±SD) from two technical replicates. Differences in expression ratios among the samples were analyzed by ANOVA (LSD test) using StatGraphics (StatPoint Technologies). Experiments for qPCR were performed at least three times.

For the microarray experiment, approximately 25 rosettes from 3-week-old noninoculated, mock-treated (1 dpi) or *P. cucumerina*-inoculated Col-0 and *aba1-6* plants (1 dpi) were collected (four biological replicates). Plant total RNA was extracted as described previously (Berrocal-Lobo et al., 2002) and further purified using the RNeasy Kit (Qiagen). RNA quality was tested by using a Bioanalyzer 2100 (Agilent Technologies). Three of the four biological replicates were independently hybridized for each transcriptomic comparison. Biotinylated complementary RNA (20 μg) was prepared, and the resulting complementary RNA was used to hybridize ATH1 Arabidopsis GeneChips (Affymetrix) using the manufacturer's protocols at the Genomic Unit of the Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas. Array images were analyzed with a GenePix 400B scanner (Molecular Devices) at 10-mm resolution. The images were quantified with GenePixPro 5.1. Gene expression levels were analyzed with GeneSpring 7.2 software (Silicon Genetics), and the chip-to-chip signal variation was minimized by normalizing signal intensities to the averaged intensity values of wild-type plants using the expression levels of the top 20th percentile of probe sets. Differentially expressed genes in untreated mutants relative to wild-type samples or in *PcBMM*-inoculated relative to the mock-treated samples were identified using two-way ANOVA and a Benjamini and Hochberg multiple testing correction (GeneSpring 7.2), as described previously (Stein et al., 2006). Genes were considered differentially expressed at $P \leq 0.01$. Up- and down-regulated genes were selected using normalized values higher than 2-fold or lower than 0.5-fold relative to control plants.

The Classification SuperViewer Tool (Bio-Array Resource, University of Toronto; http://bbc.botany.utoronto.ca/; Provart et al., 2003) was used to

generate an overview of the Gene Ontology classification of the list of differentially regulated genes in *aba1-6* and wild-type plants. The Genevestigator Meta-Analyzer tool (www.genevestigator.ethz.ch/; Zimmermann et al., 2004) was used to analyze expression profiles of differentially regulated genes in the microarray database. Hierarchical clustering of differentially expressed probe sets in *aba1-6* and wild-type plants was performed using Pearson uncentered distance, average linkage, and the Multiexperiment Viewer application from TM4 software (http://www.tm4.org/; Saeed et al., 2003).

The presence of conserved putative cis-regulatory elements in the selected genes was determined with Transplanta software (http://bioinfogp.cnb.csic.es/transplanta_dev/), which integrates a suite of bioinformatic tools (Bioprospector [http://ai.stanford.edu/~xsliu/BioProspector/], DME [http://rulai.cshl.edu/dme/], MEME [http://meme.nbcr.net/meme/], and Motif Sampler [http://bayesweb.wadsworth.org/gibbs/gibbs.html]). Subsequently, TOMTOM (http://meme.nbcr.net/meme/cgi-bin/tomtom.cgi; Gupta et al., 2007) and PLACE (http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999) were used to identify the statistically overrepresented regulatory elements in the promoters of the genes from each cluster.

Quantification of Plant Hormones

Three-week-old plants were collected before and after treatment (1 dpi) with water or a spore suspension of *PcBMM*. They were frozen immediately in liquid nitrogen and subsequently freeze dried. Tissue was then ground to a powder in a tissue lyser (Qiagen; http://www.qiagen.com/). Hormone extractions were performed on 10 mg of freeze-dried tissue as described by Forcat et al. (2008). Data are averages of three independent replicates ± SE. Statistical differences among Arabidopsis genotypes were determined by one-way ANOVA and LSD post hoc test.

Cell Wall Analysis

For FTIR, precleaned (by solvent extraction) dried leaves from at least 30 3-week-old individual plants per genotype were pooled and homogenized by ball milling. The powder was dried and mixed with potassium bromide and then pressed into 13-mm pellets. For each plant genotype, 15 FTIR spectra were collected on a Thermo Nicolet Nexus 470 spectrometer (ThermoElectric) over a range of 4,000 to 400 cm^{-1}. For each spectrum, 32 scans were coadded at a resolution of 4 cm^{-1} for Fourier transform processing and absorbance spectrum calculation using OMNIC software (Thermo Nicolet). Using win-das software (Wiley), spectra were baseline corrected, normalized, and analyzed using the principal component analysis covariance matrix method (Kemsley et al., 1996). Cell wall monosaccharides were assayed as alditol acetate derivatives (Stevenson and Furneaux, 1991) by gas chromatography performed on an Agilent 6890N gas chromatograph, and the results obtained were validated with the method described previously by Gibeaut and Carpita (1991). Myoinositol (Sigma-Aldrich) was added as an internal standard. Cellulose was determined on the fraction resistant to extraction with 2 M trifluoroacetic acid (n = 4) by phenol-sulfuric assay using Glc equivalents as standard (DuBois et al., 1956). Uronic acids were quantified using the soluble 2 M trifluoroacetic acid fraction (n = 5; Filisetti-Cozzi and Carpita, 1991). The data were analyzed using two-way Student's t test (P = 0.05). All statistical analyses were performed using the statistical software package SPSS 13.0.

Expression data from this article was deposited according to Minimum Information About a Microarray Experiment standards at ArrayExpress (EMBL-European Bioinformatics Institute) with the accession number E-MEXP-3733.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Infection of *aba1-6* and wild-type plants with the fungal transformant *PcBMM-GFP*.

Supplemental Figure S2. Callose deposition is not essential for Arabidopsis resistance to *PcBMM*.

Supplemental Figure S3. Susceptibility of the *wkry33-1* mutant to *PcBMM*.

Supplemental Figure S4. Determination of ABA, JA, and SA concentrations in leaves of wild-type plants and ABA-defective mutants.

Supplemental Figure S5. Development-associated phenotypes of 3-week-old single and double mutants defective in different hormone pathways.

Supplemental Figure S6. Biochemical analyses of the cell walls from *quadruple pyr/pyl* and *triple abi2-2* mutants and wild-type plants.

Supplemental Table S1. Genes differentially regulated in untreated (time zero) *aba1-6* plants compared with wild-type plants.

Supplemental Table S2. Genes differentially regulated in wild-type plants inoculated with *PcBMM* (1 dpi) compared with mock-treated plants.

Supplemental Table S3. Genes differentially regulated in *aba1-6* inoculated with *PcBMM* (1 dpi) compared with mock-treated plants.

Supplemental Table S4. Genes differentially regulated in untreated (time zero) *aba1-6* plants compared with wild-type plants and in wild-type plants inoculated with *PcBMM* (1 dpi) compared with mock-treated plants

Supplemental Table S5. Genes contained in the "cell wall" Gene Ontology category.

Supplemental Table S6. Genes included in clusters I to IV described in Figure 3.

Supplemental Table S7. Effect of ABA treatment on *aba1-1* resistance to *PcBMM*.

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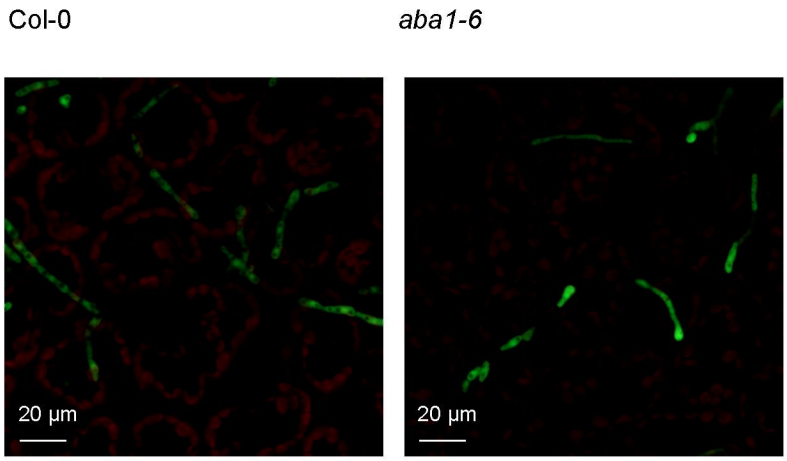
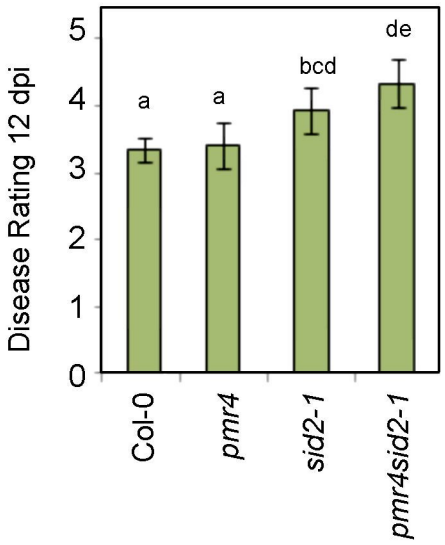
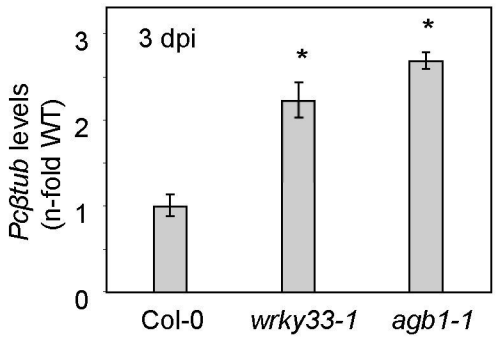


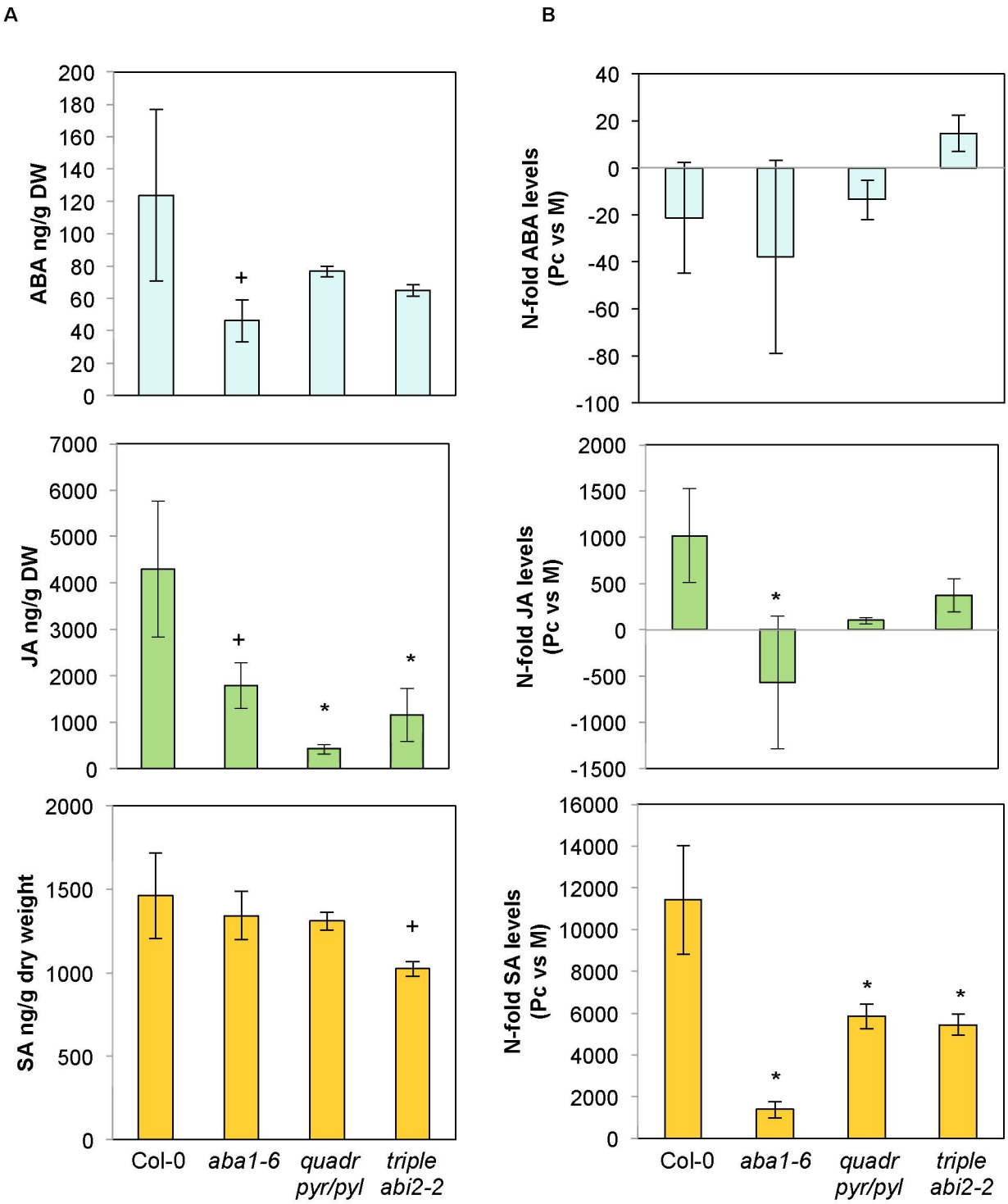
Supplemental Figure 1. *PcBMM-GFP* infection in drop-infected leaves of *aba1-6* plants



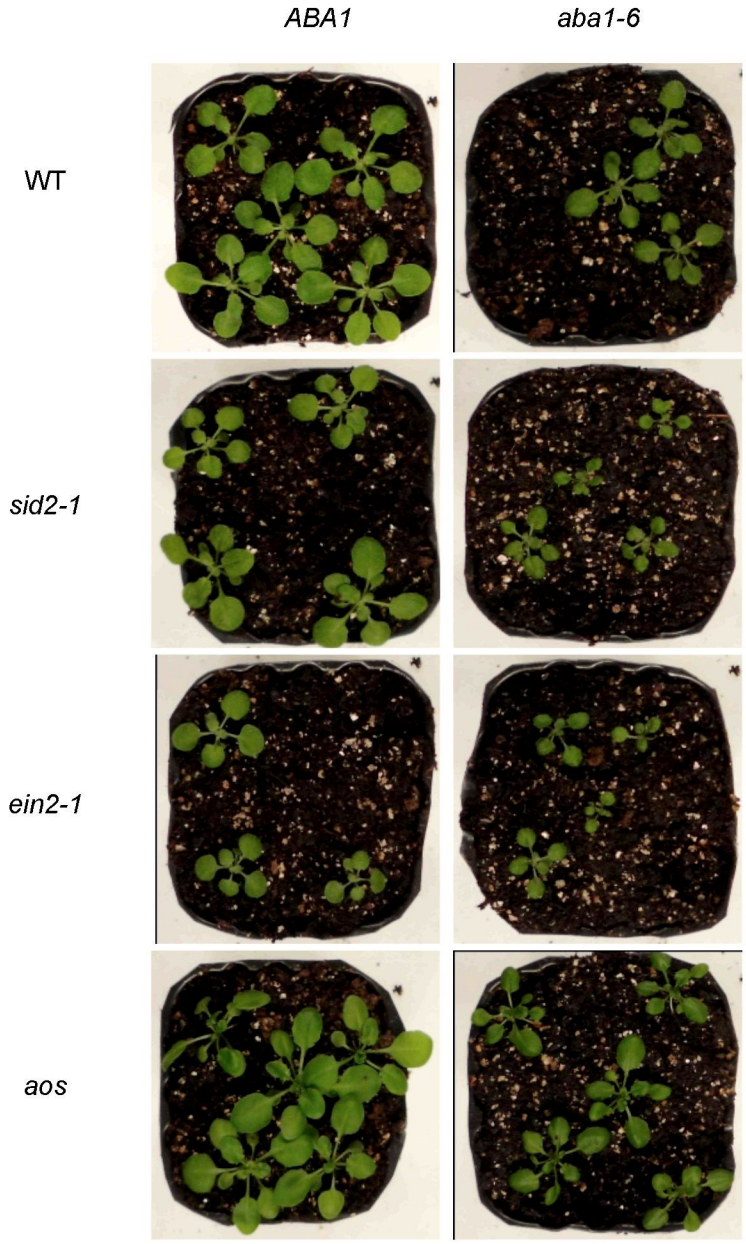
Supplemental Figure 2. Callose deposition is not essential for resistance to *P. cucumerina BMM*



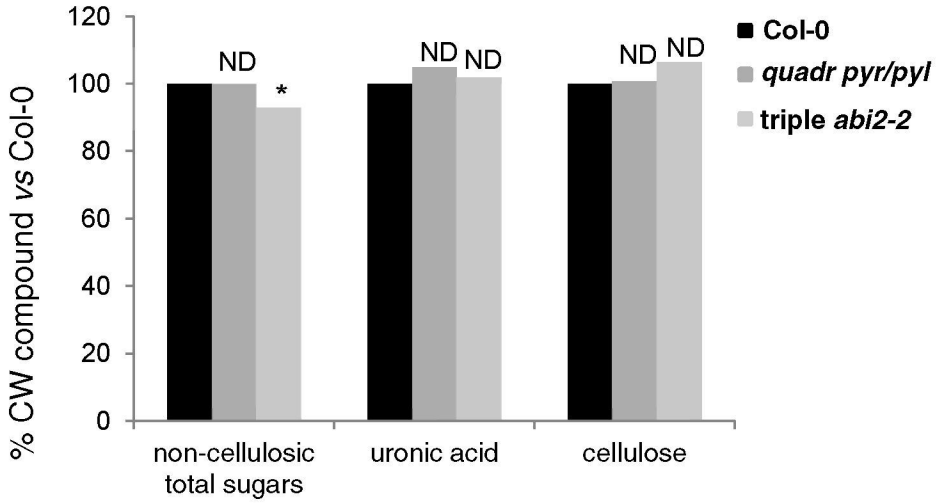
Supplemental Figure 3. Susceptibility of *wkry33-1* mutant to *PcBMM*



Supplemental Figure 4. Determination of ABA, JA and SA concentrations in leaves of wild-type plants and ABA-defective mutants



Supplemental Figure 5. Developmental-associated phenotypes of hormone defective single and double mutants



Supplemental Figure 6. Biochemical analyses of the cell walls from quadr pyr/pyl and triple abi2-2 mutants and wild-type plants.